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Prognostic Value of MIB-1 in Advanced Ovarian Carcinoma as Determined Using Automated Immunohistochemistry and Quantitative Image Analysis

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Background and Objectives: The monoclonal antibody MIB-1 is an immunohistochemical marker reacting most strongly with cells in late S phase, G_2, and M portions of the cell cycle. This antibody, reactive in formalin-fixed, paraffin-embedded tissue, allows the quantitation of a proliferation index (PI) in both current clinical cases and archival material using a computerized image analyzer (CIA).

Methods: Since many laboratories make use of automated immunohistochemistry (AIH), this study was performed to explore the technical feasibility of using AIH (Ventana ES 320) in combination with CIA (CAS 200) to evaluate MIB-1 PI as a prognostic marker as assessed by overall survival in 50 archival (formalin-fixed, paraffin-embedded), advanced stage primary ovarian carcinomas.

Results: Exploratory methods confirmed that 15% was a cutpoint that could dichotomize these 50 patients into two prognostic groups based on overall survival. The median survival of patients whose carcinoma had a high MIB-1 expression ($\geq$15%) was 16 months compared with 30 months in the patients whose tumors demonstrated low MIB-1 expression (<15%, $P = 0.01$). After adjustment for age, MIB-1 retained its prognostic significance ($P = 0.02$). Patients 60 years and older had shorter survival than younger patients ($P = 0.06$), but these two groups did not differ with respect to PI ($P = 0.76$). Those patients with a negative second look laparotomy had a longer median survival of 70 months compared with 18.5 months in patients with a positive second look ($P < 0.001$); the median PIs were 17% and 27%, respectively ($P = 0.36$). There were no significant relationships between clinical stage, nuclear grade, DNA ploidy, p53, and either survival or PI.

Conclusions: In this study, we concluded that the combination of AIH and CIA yielded a reliable quantitation of MIB-1 proliferative index and that this proliferative marker had prognostic significance in late stage ovarian carcinoma. Further studies in a larger group of patients are needed to

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confirm the relationship between proliferation index and other known clinicopathologic and genetic variables.

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INTRODUCTION

Various markers, including those of proliferative status, have been explored to improve the predictability of patient prognosis in ovarian carcinoma [1–3].

An estimate of proliferative status with preservation of tissue architecture can be performed with immunohistochemistry methodology using the monoclonal antibody Ki-67, which recognizes a nuclear antigen present in all but the resting phase (G_0) and early G_1 of the cell cycle [4]. Although the use of this antibody has been limited to studies in fresh frozen tissue, since Ki-67 is not reactive in formalin-fixed, paraffin-embeded tissue, it has provided useful prognostic information in several tumors, including ovarian carcinoma [1,3,5]. In another study of ovarian carcinoma using manual staining, Ki-67 $\geqslant$ 15% was correlated with advanced disease and shorter survival times (median 28 months compared with 18.5 months with Ki-67 <15%) [3]. In our laboratory, we performed studies using a computerized image analyzer (CIA) to quantitate a Ki-67 proliferation index (PI), defined as the ratio of immunostained nuclear area to total nuclear area, on fresh frozen tissue in advanced stage ovarian carcinomas. A PI of $\geqslant$7.5% was correlated with shorter survival times (16.8 months compared with 31.5 months with a PI of <7.5%), but PI did not correlate with histologic grade [1]. The use of Ki-67 has the disadvantage that it is limited to fresh frozen tissue. Another monoclonal antibody that marks proliferatingg cells, PCNA, reacts with formalin-fixed paraffin-embedded tissue, but it underestimates the fraction of proliferating cells since it is expressed primarily during the S phase of proliferation [6].

More recently, the monoclonal antibody MIB-1, reactive in formalin-fixed, paraffin-embedded tissue, has provided estimates of proliferative status in archival fixed tissue. MIB-1 detects the nuclear Ki-67 antigen and thus its expression is similar to that of the Ki-67 antigen. This antigen is present only in proliferating cells in the late G_1, S, G_2, or M phases and not in resting cells in the G_0 phase [7]. In breast carcinoma using CIA, the percent of cells labeled with MIB-1 on fixed sections correlated well with the percent labeling of Ki-67 on matched frozen sections. MIB-1 had advantages over Ki-67 as demonstrated by the higher percent nuclear MIB-1 staining of paraffin-embedded tissues compared with Ki-67 staining of matched frozen sections. A better quality of morphology, particularly important for CIA, was possible with paraffin compared with matched frozen sections [8]. Using CIA and formalin-fixed sections, our laboratory demonstrated probabilities for 5-year survival of 0.95, 0.8, and 0.39 with MIB-1 PI cutpoints of <5%, 5–11%, and >11%, respectively, in early stage breast carcinoma [9].

Only a few studies have explored the use of MIB-1 in ovarian carcinoma. In advanced stage ovarian carcinomas, using CIA on fixed sections, our laboratory showed that an MIB-1 PI of $\geqslant$7% was correlated with shorter survival times of 16.5 months compared with 33.2 months when the PI was <7% [2]. These results compared well with our previous study using Ki-67 on matched frozen tissue from a similar cohort of patients [1]. Other investigators reported a significant relationship between lower PI and disease-free survival in a study of stage III carcinoma of the ovary using MIB-1 and fixed tissue. They reported a survival advantage when the MIB-1 PI was less than 19.2% [10]. In a case report of a stage III ovarian clear cell carcinoma and fresh frozen tumor, an increase in the initial MIB-1 PI from 4.1% to 38.7% upon recurrence prompted the investigator to suggest further studies exploring whether more aggressive therapy in such patients is warrented [11].

In the clinical laboratory and in clinical research studies, there has been expanding use of automated immunohistochemistry (AIH) to increase productivity and avoid possible inconsistencies in staining and thus in interpretation of manual immunohistochemistry. In preparation for this newer technology, we investigated the technical feasibility of AIH compared with manual staining, in combination with CIA for the determination of MIB-1 PI in a series of 50 advanced ovarian carcinomas. Assessment and correlation of these results with clinical stage, histologic grade, DNA ploidy, age, p53 level, second look outcome, and survival were performed to evaluate this method as a prognostic factor that may allow for individualization of postoperative treatment in advanced ovarian carcinoma.

MATERIALS AND METHODS

Patients

Archival paraffin-embedded tissue blocks from 50 patients with advanced stage primary ovarian carcinoma, as determined by criteria established by the International Federation of Gynecologists and Obstetricians (FIGO) were selected for this study [12,13]. These patients un-

derwent exploratory laparotomy on the Gynecologic Oncology Service at Duke University Medical Center between 1985 and 1990 as part of their initial staging for primary epithelial ovarian cancer. These cases were from a cohort previously studied in our laboratory [1,2]. At the time of surgery, representative portions of the tumors were bisected, with half of the sample flash frozen and the other half formalin-fixed and paraffin-embedded. All histologic grading was derived from reviewing the entire set of permanent slides, using criteria as described by Russell and Bannatyne [14]. The material from these 50 cases was reviewed by a single pathologist (L.J.L.).

DNA Ploidy by Cellular Image Analysis

DNA ploidy was performed as previously described [15]. In brief, touch preparations were made from the fresh frozen tissue blocks and incubated with Feulgen dye; tumor cells were then analyzed for ploidy using the CAS 200 image analysis system and the Quantitative DNA Analysis software (QDA version 3.0) (Becton Dickinson Cellular Imaging Systems, San Jose CA).

Primary Antibodies

The proliferation monoclonal antibody used in this study was MIB-1 (AMAC, Westbrook ME) which was diluted 1:100 in pH 7.4 phosphate-buffered saline containing 2% bovine serum albumin (2% BSA/PBS), as previously described [2,9]. Normal mouse IgG_1 (Coulter Source, Marietta, GA) was diluted 1:100 in 2% BSA/PBS and used as a negative antibody control. Anit-cytokeratin cocktail AE1/AE3 (Boehringer Mannheim, Indianapolis IN) was diluted 1:50 in 2% BSA/PBS and used as an antigenicity control. This cocktail of antibodies reacts specifically with and localizes both high and low molecular weight human epithelial cytokeratins [16]. Staining with MIB-1 was performed by AIH and determination of the PI was performed by cellular image analysis (CIA) as described below.

The manual staining of paraffin-embedded tissue with p53-specific monoclonal antibody (Ab-2, Oncogene Sciences, Manhasset, NY) used in this study has been previously described [17]. P53 overexpression was scored as positive only if definite nuclear staining was clearly present in >25% of the malignant cells.

Automated Immunohistochemistry (AIH) for MIB-1 Antibody

Sample preparation and microwave antigen retrieval from the paraffin sections were performed as previously described [2]. Upon completion of the cooling process subsequent to microwave antigen retrieval, the slides were washed in two changes of wash buffer solution (Ventana), and the appropriate bar code label was applied to each slide. The slides were then attached horizontally by metal clips on a level carousel within the temperature equilibrated reaction chamber. The Ventana ES 320 was activated by loading the pre-programmed MIB-1-IHC recipe file. Each recipe file consists of a specific sequence of buffer rinses, enzyme inhibitors, blocking serums, antibodies, detection complexes, chromogens, and counterstains.

After the initial series of buffer rinses and normal serum preincubations, MIB-1, IgG_1, and AE1/AE3 primary antibodies were dispensed using a 100 μl metered dose per slide volume mechanical plunger. The specific antibodies were localized by a universal anti-rabbit and anti-mouse secondary IgG-biotinylated antibody cocktail (Ventana). This step was followed by a streptavidin-enzyme conjugate and visualized as a brown stain with diaminobenzidine (DAB) chromogen with copper sulfate enhancement. Each step was incubated for a precise amount of time and at 42°C standardized temperature. At the end of each incubation step, the instrument rinsed the sections to stop the reaction and remove unbound material that could potentially cause nonspecific background staining. Following the automated staining process, the slides were rinsed in tap water followed by sodium acetate incubation and then counterstained with 1.5% methyl green for 5 min. The nuclear counterstain methyl green was chosen because it provides the best spectral separation from the brown DAB chromogen [18]. Subsequent to counterstaining, the slides were dehydrated in acetone and cleared in xylene. The slides were then coverslipped and labeled.

Determination of Proliferation Index by Cellular Image Analysis (CIA)

The proliferation index using MIB-1 was quantitated using a CAS 200 Image Analysis System in combination with the Quantitative Proliferation Index CAS Software Program, QPI, version 2 (Becton Dickinson Cellular Imaging Systems), as previously described [9]. Data obtained by computer-assisted image analysis was expressed as the PI, defined as the percent of nuclear area positively stained with the MIB-1 monoclonal antibody relative to the total nuclear area stained with methyl green. At a magnification of 400×, 15 representative fields of the tumor, generally 1,000–2,000 cells, were analyzed. After 15 fields, the coefficient of variation of the accumulative measured proliferation index had usually reached a statistical plateau. Serial control sections, stained with normal mouse IgG_1, were prepared in each case, observed by standard microscopy, and analyzed to establish background immunostaining thresholds.

The scene segmentation available using QPI allows MIB-1 antibody reactivity to be quantitated only in areas

TABLE I. Relationship Between Clinicopathologic Variables and Survival and MIB-1 Proliferation Index*

Variable	Category	No.	Median survival (95% CI), months	*P* value	Median PI (range)	*P* value
Age (yr)	<60	24	19.0 (15–37)	0.06	25 (2–63)	0.76
	⩾60	26	17.7 (16–24)		19 (0.8–77)	
Clinical stage	III	35	18.5 (16–24)	0.44	20 (0.8–63)	0.21
	IV	15	16.8 (15–33)		29 (6–77)	
Nuclear grade	1 and 2	21	17.5 (13–33)	0.53	17 (0.8–77)	0.37
	3	29	18.5 (16–25)		25 (2–63)	
DNA ploidy	Euploid	12	18.0 (15–53)	0.14	19 (4–49)	0.17
	Aneuploid	38	17.7 (16–24)		22 (0.8–77)	
p53	Negative	21	20.0 (16–37)	0.14	17 (0.8–63)	0.30
	Positive	29	17.3 (15–22)		23 (2–77)	
Second look	Negative	7	70.0 (37–[a])	<0.001	17 (2–63)	0.36
	Positive	18	18.0 (16–24)		27 (0.8–63)	

*CI, confidence interval; PI, proliferation index.
[a]Upper limit not calculable; see statistical methods.

containing carcinoma cells, eliminating areas of normal or lymphoid tissue.

Statistical Analysis

Since other studies have reported a relationship between increased proliferation value and poorer clinical outcome, we sought to establish this relationship for MIB-1 with respect to survival and to suggest prognostic cutpoints for MIB-1 that could be clinically useful. Survival was defined to be the interval from diagnosis of ovarian cancer to death or last follow-up time, where patients still alive at last follow-up were right-censored in the survival analyses. Survival probabilities were estimated by the method of Kaplan and Meier [19]. Differences in survival between groups were tested with the log rank statistic [20]. Confidence intervals for median survival time were calculared by the method of Brookmeyer and Crowley [21]. Occasionally, when the last death time does not satisfy the definition for the confidence interval, the upper confidence limit cannot be obtained. Median follow-up time was estimated from the survival times by reversing the role of the censoring indicator in a Kaplan-Meier analysis.

Two approaches were used to assist in the development of a potentially useful prognostic cutpoint. The first approach used tertiles of the frequency distribution of MIB-1 values to define three groups of nearly equal size. The second approach was based on maximizing differences in the estimated hazard ratios for groupings of the MIB-1 values [22]. This exploratory analysis technique necessarily gives the lowest possible ''*P* value(s)'' attainable, and hence statistical significance should not be interpreted in the usual way. A multivariate analysis was performed to assess (by likelihood procedures) whether the addition of MIB-1 to a Cox regression model consisting of known prognostic variables significantly improved the fit of the model [23].

Differences in median MIB-1 values between categories of clinicopathologic variables were tested using rank sum methods [24]. All statistical tests were two-sided and were carried out at the 0.05 level of significance.

RESULTS

The patient population had a median age of 60 years (range, 30–80 years) and comprised 35 (70%) stage III carcinomas and 15 (30%) stage IV carcinomas. The histologic types consisted of 45 (90%) papillary serous and five (10%) mucinous tumors. There was 1 (2%) grade 1 tumor, 20 (40%) grade 2 tumors, and 29 (58%) grade 3 tumors. Twelve (24%) cases were euploid and 38 (76%) were aneuploid. p53 expression was observed in 29 (58%) cases. Second look laparotomy was performed on 25 patients and resulted in a negative assessment in 7 (28%) cases. After a median follow-up time of 8 years, only five patients remained alive (from 3 to 10 years) at the cutoff date for the analysis of December 1996.

MIB-1 immunostaining usually demonstrated a heterogeneous staining pattern throughout any given tumor section. The coefficient of variation of the cumulative measured proliferation index generally reached a statistical plateau for computerized CIA when 15 fields had been counted.

The median MIB-1 PI for all patients was 22% (range, 0.8–77%). In addition to MIB-1, the variables analyzed include age (<60, ⩾60 years), clinical stage (III, IV), nuclear grade (1, 2, 3), DNA ploidy (euploid, aneuploid), p53 (+,−), and second look laparotomy outcome (+,−). The relationship between these additional variables and survival, as well as PI, is given in Table I. Patients ⩾60

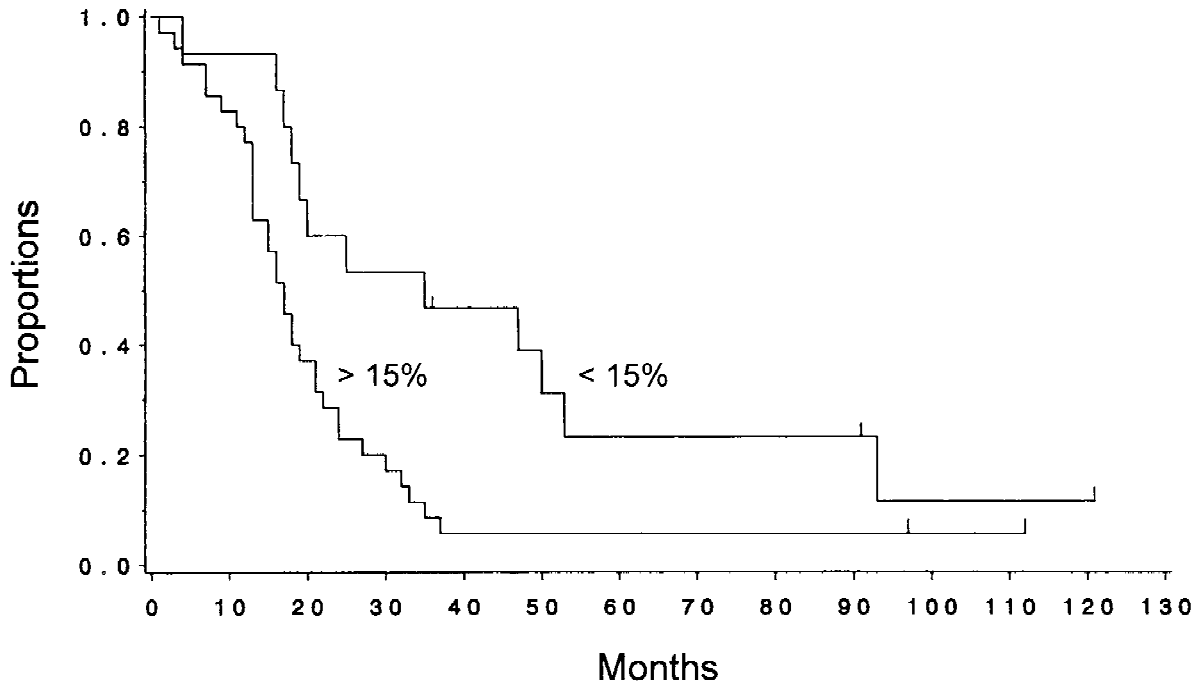

Fig. 1. Relationship between the proliferation index determined by MIB-1 analysis and survival in advanced epithelial ovarian cancer ($P = 0.01$). The median survival was 30 months for those patients with low MIB-1 expression (<15%) and 16 months for those whose tumors demonstrated a high MIB-1 expression (⩾15%).

years had a shorter survival than younger patients ($P = 0.06$), and patients with a negative second look survived significantly longer than those with a positive second look ($P < 0.001$). None of the other additional variables appeared to be related to survival. There were no significant differences in median PI between any of the categories tested in Table I.

To examine the relationship between MIB-1 and survival, three groups were created based on tertiles of the frequency distribution: <15% (n = 15), 15–29% (n = 17), and ⩾30% (n = 18). The median survival times for these three groups were estimated to be 30 months, 18 months, and 13 months, respectively ($P = 0.03$). In addition, exploratory methods were used based on maximizing differences in the estimated hazard ratios for two or more groups. This approach further suggested that 15% was a potentially useful value for dichotomizing patients according to risk. Patients with MIB-1 PI ⩾15% had an estimated median survival of 16 months compared with 30 months for those with an MIB-1 PI <15% (Fig. 1; $P = 0.001$). The relationship between MIB-1 and survival retained significance after adjusting for age in a Cox regression analysis ($P = 0.02$). In the group of 25 patients with second look laparotomy results, MIB-1 PI ⩾15% had a positive predictive value of 78% for a positive second look outcome and a PI <15% had a negative predictive value of 43% for a negative second look outcome.

DISCUSSION

In this study, we evaluated the technical feasibility of AIH in combination with CIA to enumerate the fraction of nuclei immunostaining with MIB-1 and to calculate the PI to determine its usefulness as an independent prognostic indicator of survival in a series of 50 advanced stage primary ovarian carcinomas. As demonstrated by the statistical plateau of the coefficient of variation of the accumulative measured PI in this (data not shown) and previous studies, and by the absence of statistically significant intra- or interobserver variation in the measured MIB-1 PI in previous studies, this methodology yielded a reliable assessment of tumor proliferative activity [9]. Subjectivity may have significant impact on the evaluation of the MIB-1 index by image analysis in that fields and cells chosen for analysis must be manually selected. These issues have been addressed in prior studies where interobserver and intraobserver variability were low [9,25]. In our laboratory the Pearson correlation coefficient for intraobserver measurements of PI has been 0.99 and for interobserver measurement 0.98 [25].

Several studies have suggested that proliferative status may provide useful prognostic information in ovarian carcinoma. These various methodologies have included the determination of DNA thymidine labeling index [26,27], immunohistochemical detection of bromodeoxyuridine (BrdU) incorporation into DNA of cultured tumor cells [28], flow cytometric determination of S-phase

[28], flow cytometric determination of DNA ploidy [29,30], CIA determination of DNA ploidy in touch imprints of frozen tumor [15], and manual immunohistochemical detection with manual semi-quantitative or CIA determination of Ki-67 staining in fresh frozen tissue [1,3,5]. Disadvantages of these methods include loss of tissue morphology and architectural features, requirement for fresh frozen tissue, use of radioisotopes, and lack of method standardization. Manual immunohistochemical staining and manual semi-quantitative determination of MIB-1 PI in paraffin-embedded ovarian tumors have been reported and appear to overcome most of these disadvantages [10]. However, these manual methods have the potential disadvantages of difficulty in standardization and inconsistency of staining. Studies using CIA in advanced ovarian carcinoma using Ki-67 in frozen tissue and MIB-1 PI in paraffin-embedded tissue have allowed for more standardization, but the potential inconsistency of manual staining remains [1,2].

A prior study of a different series of 50 primary advanced stage ovarian carcinomas in our laboratory using manual immunohistochemistry and CIA to quantitate MIB-1 PI suggested that a threshold PI of 7% could stratify patients into statistically significant prognostic groups as determined by overall survival. The median MIB-1 PI overall was 14.3% (range, 0.3–44.3%) in that study [2]. In the present study, using AIH and CIA to quantitate MIB-1 PI in a similar cohort of 50 primary advanced stage ovarian carcinomas, the median MIB-1 was 22%, slightly higher than in the previous study. Correspondingly, the prognostic cutpoint of 15% (in the present study) was somewhat higher but did divide patients into favorable and unfavorable prognostic groups ($P = 0.01$). As in the previous study, a somewhat higher median PI of 29% was observed in stage IV tumors relative to a lower median PI of 20% in stage III tumors. When patients were stratified by the potential prognostic features of clinical stage, nuclear grade, age, p53 expression, or DNA ploidy status, there was no statistically significant relationship with survival. Similar stratifications showed no statistically significant correlations with PI. Stratification by second look findings showed a relationship to overall survival.

Other investigators have explored the use of stratification of patients into high and low recurrence risk groups as an aid in patient selection to improve second look laparotomy ''accuracy'' (defined as the percent of negative second look patients with no subsequent recurrence after 2 years of subsequent follow-up). Further analysis in this risk stratification study of the data from the second look outcome prior to the 2 year follow-up period by using methods described by Galen and Gambino [31] showed a 77% positive predictive value of high-risk criteria (ability of high-risk criteria to predict a positive second look) and a 39% negative predictive value of low-risk criteria (ability of low-risk criteria to predict a negative second look). Subsequent to the 2 year follow-up, the investigators reported only a 9% ''accuracy'' of the second look negative results in the group defined by high-risk criteria, but a 66% ''accuracy'' in the group defined by low-risk criteria, leading the authors to suggest that second look laparotomy should be limited to the patients with low-risk criteria. Using the cutoff of 15% MIB-1 PI obtained in the current study, MIB-1 had predictive values for second look outcome similar to those reported above using clinical risk factors. Although only 25 patients has second look outcome results, these preliminary results suggest that the consideration of MIB-1 PI in addition to clinical risk factors might further improve the selection of patients who would be more likely to benefit from a second look procedure.

In this study we concluded that AIH is technically feasible in combination with CIA for the determination of MIB-1 PI, that this proliferative marker has prognostic value in advanced ovarian carcinoma, and that inclusion of this marker with clinical risk criteria may improve patient selection for second look laparotomy. Further studies in a larger group of patients are needed to confirm these results.

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COMMENTARY

After the cell cycle was described in 1953 by Howard and Pelc [1] and tritiated thymidine was introduced as a highly specific marker for DNA synthesis [2], enthusiasm for applying cell kinetics techniques to tumors was great. This led to rapid development of the field of cell kinetics, but practical clinical applications developed slowly. The principal technique was tritiated thymidine labeling, which required administration of the radioisotope tritium to subjects or in vitro incubation of tissue to determine S-phase fractions (SPF). Prolonged in vivo use is necessary to measure growth fraction [3], meaning the fraction of cells in any phase of the replicative cycle, not just in S. In vitro labeling with tritiated thymidine or its surrogate, 5-bromodeoxyuridine, gives precise and clinically useful SPF measurements, but these techniques have not been applied extensively because of technical requirements. Flow cytometric DNA measurements were initially widely touted because of a high level of automation. DNA flow cytometry did not meet expectations because of difficulties in preparing cell suspensions from solid tumors and inability to exclude contaminating nonmalignant cells from the measurements. While flow cytometric SPF measurements were clinically predictive [4], some specimens were not measurable and in others large errors occurred [5]. This led to reluctance to use flow cytometric SPF in clinical decisions.

Discovery of the Ki-67 antigen, which is expressed throughout the cell cycle and therefore represents growth fraction, offered a new approach but was hamptered by the requirement for fresh or freshly frozen tissue for the initial antibody [6]. Subsequent development of other antibodies have resolved this problem, making the antigen detectable in formalin-fixed, paraffin-embedded tissues [7]. Several papers have appeared recently using the MIB-1 antibody against Ki-67, and results suggest that it can be used successfully by ordinary laboratories capable of carrying out immunohistochemistry. Nonetheless, studies of reproducibility must be carried out. Layfield and associates in this issue of the Journal have shown the practicality of applying automation of staing and image analysis to Ki-67 antigen detection with MIB-1. Their technique makes subjective selection of fields in the measurement of K1-67 index unnecessary, thereby removing operator-induced bias. Laboratories using their methods could readily compare results. It seem likely that at last a widely applicable, reproducible, dependable, and accurate method for measuring cellular proliferation in human tumors may have been achieved. Furthermore, Layfield et al. demonstrated the prognostic power of the Ki-67 index in high-grade ovarian carcinoma in multivariate analysis, adding that tumor to a list that includes breast carcinoma, sarcomas, malignant lymphomas, leukemias, astrocytomas, and meningiomas for which proliferative indices are clinically predictive.

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